

REMARKS

This Amendment, filed in reply to the Office Action dated May 23, 2008, is believed to be fully responsive to each point of objection and rejection raised therein. Accordingly, favorable reconsideration on the merits is respectfully requested.

Claims 16-18 are rejected. Claims 16-18 are amended herewith to further clarify Applicants' claimed invention. Support for the recited method steps in Claims 16-18 can be found throughout the specification as filed, and at, for example, pages 39-42 of the specification as filed, and in the "comparison of expressed amounts in normal colon and colon cancer cell" example set forth on pages 55 and 56 of the specification as filed. Support for recitation of "at least 99.29% identical" in Claims 16-18 can be found in the paragraph bridging pages 23 and 24, and the first full paragraph of page 24, of the specification as filed. No new matter is added by way of this amendment. Entry and consideration of this amendment are respectfully requested.

Claim to Priority

Applicants thank the Examiner for acknowledging Applicants' claim to foreign priority, and receipt of Applicants' foreign priority document from the International Bureau, namely Japanese Patent Application No. 2003-341245.

Information Disclosure Statements

Applicants thank the Examiner for returning signed and initialed copies of the PTO Forms SB/08 that accompanied the Information Disclosure Statements filed March 30, 2006, and July 25, 2006, indicating consideration of the references cited therein.

Drawings

Applicants thank the Examiner for acknowledging acceptance of the drawings submitted March 30, 2006.

Objections to the Claims

1. On page 4 of the Office Action, Claims 16-18 are objected to as depending from non-elected claims.

Applicants note that Claim 16 has been amended so as to be placed in independent form, mooted the objection.

2. On page 4 of the Office Action, Claims 17 and 18 are objected to for recitation of “[t]he judging method.” The Examiner suggests amending the claims to recite “[t]he method.”

Solely to advance prosecution, and without agreeing with the objection, Applicants herewith amend Claims 17 and 18 to recite “[t]he method ...” Applicants respectfully submit that the amendment overcomes the objection.

3. On page 4 of the Office Action, Claims 16-18 are objected to for recitation of “DNA represented by.” The Examiner suggests amending the claims to recite “DNA comprising.”

Applicants note that Claims 16-18 as amended do not recite “DNA represented by,” thus the rejection is moot.

4. On page 4 of the Office Action, Claims 16-18 are objected to for recitation of “nucleotide sequence described in SEQ ID NO: 1.” The Examiner suggests amending the claims to recite “nucleotide sequence of SEQ ID NO: 1.”

Solely to advance prosecution, and without agreeing with the objection, Applicants herewith amend Claims 16-18 to recite “nucleotide sequence of SEQ ID NO: 1.” Applicants respectfully submit that the amendment overcomes the objection.

Withdrawal of the objections is respectfully requested.

Claims 16-18 are Proper Under 35 U.S.C. § 112, Second Paragraph

1. On page 5 of the Office Action, Claims 16-18 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite. Specifically, the Examiner contends that Claims 16 and 17 are indefinite for failing to recite *any* steps performed in the claimed method. Claim 18 is rejected as being dependent on Claim 17.

Applicants note that Claims 16-18 have been amended to recite positive method steps, including a measuring step and a correlative step. Support for recitation of these method steps can be found throughout Applicants’ disclosure as originally filed, and at, for example, pages 39-42 of the specification as filed, and in the “comparison of expressed amounts in normal colon and colon cancer cell” example set forth on pages 55 and 56 of the specification as filed. Applicants respectfully submit that the amendments overcome the rejection.

2. On page 5 of the Office Action, Claims 16-18 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite. Specifically, the Examiner contends that recitation of “characterized in that” in Claim 16 is unclear.

Applicants note that Claims 16-18 as amended do not recite “characterized in that,” thus the rejection is moot.

3. On page 5 of the Office Action, Claims 16-18 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite. The Examiner contends that recitation of “judging whether or not,” as recited in Claim 16, is vague, as it is unclear what steps are encompassed by the term “judging.”

Applicants note that Claims 16-18 as amended do not recite “judging whether or not,” thus the rejection is moot.

Withdrawal of the indefiniteness rejections is respectfully requested.

Claims 16-18 are Adequately Described Under 35 U.S.C. § 112, First Paragraph

On page 6 of the Office Action, Claims 16-18 are rejected under 35 U.S.C. 112, first paragraph, as lacking an adequate written description.

In making the rejection, the Examiner asserts that Claims 16-18 encompass the use of *any* nucleotide sequence with or without relation to the encoded protein of SEQ ID NO: 1, by virtue of the recitation in Claim 4 (upon which Claims 16-18 ultimately depend) that the DNA detected by the claimed method “comprises a nucleotide sequence wherein 1 or 2 or more of bases in the DNA sequence ... are deleted, substituted or added.”

Solely to advance prosecution, and without acquiescing in the rejection, Applicants herewith amend Claim 16 to recite that the measured RNA is the product of transcription of a nucleotide sequence selected from the group consisting of (a) and (b), wherein nucleotide sequence (a) is a DNA comprising the nucleotide sequence of SEQ ID NO: 1, and wherein nucleotide sequence (b) is a DNA comprising a nucleotide sequence at least 99.29% identical to positions 94 to 2934 of SEQ ID NO:1, and which encodes a protein having a cell growth accelerating activity. Applicants note that recitation of the phrase “99.29% identical” finds

implicit and inherent support in the paragraph bridging pages 23 and 24, and the first full paragraph of page 24, of the specification as filed. Specifically, line 10 on page 24 recites that “[the 1 or 2 or more bases deleted, substituted or added] is generally from 1 to 20.” Applicants note that the 2840 base pair polynucleotide sequence of positions 94-2934 of SEQ ID NO: 1, *in which 20 nucleotides have been deleted, substituted or added*, results in a polynucleotide sequence at least 99.29% identical to SEQ ID NO: 1. Applicants respectfully submit that one of skill in the art would readily understand which residues of SEQ ID NO: 1 are required for activity, and similarly which residues are dispensable for activity, by multiple-alignment analysis of the nucleotide sequences of known C1-THFS isozymes, thus arriving at the structure-function relationship amongst C1-THFS isozymes. Applicants note that to comply with the written description requirements of section 112, first paragraph, the description need only describe in detail that which is new or not conventional. See *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d at 1384, 231 USPQ at 94. Multiple nucleotide sequence alignment to identify common conserved regions and structures amongst homologues was conventional in the art as of Applicants’ filing date, as was the existence of point mutations within conserved genes from different individuals. Accordingly, one of skill in the art would have understood Applicants to be in possession of the invention as claimed, thus the written description requirement is met.

Withdrawal of the written description rejection is respectfully requested.

Claims 16-18 are Enabled Under 35 U.S.C. § 112, First Paragraph

On page 7 of the Office Action, the Examiner rejects Claims 16-18 under 35 U.S.C. §112, first paragraph, as lacking enablement. Specifically, the Examiner states that the specification is enabling for a method of diagnosing colon cancer by detecting nucleic acid

molecules of a nucleotide sequence “from SEQ ID NO: 1 or from the 94th to 2934th base pairs of SEQ ID NO: 1.” However, the Examiner contends that the specification does not reasonably provide enablement for a method of diagnosing colon cancer by detecting *any* nucleotide sequence of *any* protein, wherein one or more bases are deleted, substituted or added to the nucleotide sequence of SEQ ID NO: 1.

Further, the Examiner alleges that neither the specification nor the art provides a correlation between structure and function and disease state, such that one of skill in the art would be able to envision the structure of any nucleotide sequence with the claimed alterations. The Examiner also asserts that the art discloses that modification of a polypeptide sequence, with preservation of biological function, is highly unpredictable. Specifically, the Examiner argues that at the time of the invention there was a high level of unpredictability associated with altering a polypeptide sequence with the expectation of maintaining the desired biological activity, citing Branden *et al.*, Witkowski *et al.* and Seffernick *et al.*

The Examiner concludes that the claims lack enablement because the specification does not establish (1) regions of the protein structure which may be modified without affecting enzymatic activity, (2) the general tolerance of the claimed polypeptide to modification, and (3) a rational and predictable scheme for mutating the claimed polynucleotides to arrive at a sequence encoding a protein with the desired biological function.

Initially, Applicants respectfully disagree that it would constitute undue experimentation to use the claimed polynucleotide sequence as a marker to identify colon cancer. Nevertheless, Applicants note that Claim 16 has been amended to recite that the measured RNA is the product of transcription of a nucleotide sequence selected from the group consisting of (a) and (b), wherein nucleotide sequence (a) is a DNA comprising the nucleotide sequence of SEQ ID NO: 1,

and wherein nucleotide sequence (b) is a DNA comprising a nucleotide sequence at least 99.29% identical to positions 94 to 2934 of SEQ ID NO:1, and which encodes a protein having a cell growth accelerating activity.

In view of the amendments to the claims attached herewith, Applicants disagree that it would be undue experimentation for one of skill in the art to determine which of the claimed polynucleotide variants encode proteins possessing the requisite biological activity, and thus be used as a marker to identify colon cancer cells. Specifically, Applicants note that the extraction, sequencing, alignment and mutation of DNA molecules was routine as of Applicants' filing date. Furthermore, the instant specification, in the example entitled "[C]ell-growth Enhancing Activity of Protein Encoded by the Gene Concerned in the DNA to be provided by the invention" as set forth on pages 59 and 60 of the specification as filed, clearly describes an assay which can be used to determine whether a variant of SEQ ID NO: 1 encodes a protein possessing the requisite biological activity, and one of skill in the art would understand as such. Consistent with relevant case law, even extended experimentation is not undue if the skilled artisan is given sufficient direction or guidance." See *In re Colianni*, 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977). Further, it is also well-settled that a considerable amount of experimentation does not preclude a finding of enablement, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed. See *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) and *In re Angstadt*, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976). Clearly, Applicants' disclosure as filed provides for all of the above. Accordingly, practicing Applicants' claimed invention would require no more than routine experimentation.

Further, regarding the alleged unpredictability of which nucleotides or amino acids may be substituted and which need to be conserved to maintain “enzyme activities,” Applicants again point out that it was routine in the art to align homologous sequences from different species, using any of the alignment algorithms well known in the art as of Applicants’ priority date, to generate a consensus sequence. One of skill in the art would understand that a consensus sequence indicates the most common nucleotide or amino acid at each position and that regions of proteins containing strong consensus sequences typically represent conserved functional domains or structural elements. These conserved functional domains are often essential for the functioning of a protein, hence their conservation. Accordingly, using homologous nucleotide or amino acid sequences and alignment algorithms well-known in the art prior to Applicants’ effective filing date, one of skill in the art can gain an understanding as to what nucleotides or amino acids are likely to be essential for “enzyme activities.” Put another way, the relationship between the nucleotide or amino acid sequence and the function of a protein is evident in view of the evolutionary conservation amongst homologues, and one of skill in the art would be able to deduce, using the guidance provided by the instant specification, without undue experimentation, residues that should be conserved to preserve the “enzyme activities.”

Further still, Applicants point out that one of skill in the art would be well-apprised of the amino acids that correlate with the biological activities of the claimed polypeptides. By way of example, the specification discloses that the biological activities of human C1-THFS (an isozyme of the polypeptide encoded by SEQ ID NO: 2) “are not easily lost by a change ...

caused by a DNA mutation” (page 20). Similarly, Barlowe *et al.*¹ (*Biochem.*, 1989, 28:2099-2106) state that “[s]ite specific mutations in the dehydrogenase/ cyclohydrolase domain [of yeast C1-THFS] did not affect synthetase activity, consistent with the proposed domain structure . . . [where] certain mutations actually increased the cyclohydrolase catalytic activity. Abstract. Accordingly, from the knowledge in the art correlating amino acid sequence to structure and function of C1-THFS isozymes, Applicants submit that the specification as filed, coupled with information known in the art, provide adequate guidance to allow one of skill in the art to determine which amino acids in the polypeptide sequence are tolerant of modification, without undue experimentation. Nevertheless, even if *some* experimentation is required to determine which variants possess the requisite biological activities, such experimentation would be merely routine, and not undue, particularly because the claims as amended recite that the variants are at least 99.29% identical.

Further still, Applicants respectfully submit that, contrary to what they are cited for, Branden *et al.*, Witkowski *et al.*, and Seffernick *et al.*, actually serve to demonstrate the predictability of nucleotide or amino acid substitution on protein function. As an example, Branden *et al.* disclose that protein engineering is actually an attempt to mutate a gene “in a predictable way.” Further, Witkowski *et al.* disclose that specific mutations were “entirely consistent with [their] observation that introduction of [a specific] side chain . . . creates a high-affinity binding site,” where the authors believe that they have elucidated a “valuable paradigm.” Page 11648. Finally, Seffernick *et al.*, which appears to have been relied upon to support the

¹ In accordance with M.P.E.P. 609(c), the documents cited herein in support of Applicants’ remarks are being submitted as evidence directed to an issue raised in the Official Action, and no fee pursuant to 37 C.F.R. 1.97 or 1.98, or citation on a FORM PTO/SB/08 or PTO-1449 is believed to be necessary.

contention that identical nucleotide sequences can elicit various protein functions, actually discloses that protein-family members “that catalyze different reactions are generally divergent to the extent that amino acid sequence identity is less than 50%.” Thus, Seffernick *et al.* demonstrates that their disclosed, contrary results are anomalous.

Furthermore, as evidence that it would not require undue experimentation to practice Applicants’ invention as claimed, Applicants refer the Examiner to the Board’s decision in *Ex parte Kubin*, in which claims directed to “polypeptides at least 80% identical to amino acids 22-221 of SEQ ID NO:2” were found to be fully enabled. In making such a determination, the Board acknowledged that the specification did not disclose any variant of SEQ ID NO:2 within amino acids 22-221. However, the Board acknowledged the high skill level in the field, and that the methods for making and screening the variant sequences were known in the art, and concluded that “the experimentation involved to produce other sequences within the scope of the claims would have been well within the skill of those in the art,” and thus routine. Although the Board acknowledged that the amount of experimentation to practice the full scope of the claimed invention might have been extensive, it would have been routine, particularly when considering the advanced state of the art, and the relative skill of those in the art. Thus, relevant case law clearly indicates that the instant rejection does not reasonably consider the skill and knowledge possessed by one of skill in the art.

Lastly, Applicants refer the Examiner to the working example disclosed on page 56 of the specification as filed, which discloses an experiment which clearly demonstrates an increased amount of the transcripts of the DNA of the present invention in colon cancer cells, which is depicted in Figure 4.

In view of the foregoing, Applicants respectfully submit that the claims as amended fully comply with the enablement requirements of section 112, first paragraph.

Withdrawal of the enablement rejection is respectfully requested.

Claims 16-18 are Patentable Under 35 U.S.C. § 102

On page 12 of the Office Action, the Examiner rejects Claim 16 under 35 U.S.C. 102(b) as being anticipated by Tang *et al.* (WO 2002/83873).

The Examiner alleges that Tang *et al.* disclose a gene encoding a protein holoenzyme complex tetrahydrofolate synthase having the aforementioned “enzyme activities,” which is 100% identical to instant SEQ ID NO: 1. The Examiner further asserts that Tang *et al.* disclose that the gene is expressed in cancer tissue including colon carcinoma, and that diagnosis may be achieved by detecting the expressed nucleic acid molecule. The Examiner concludes that Tang *et al.* anticipates Claim 16.

Applicants respectfully disagree, and traverse the rejection on the following grounds.

Initially, Applicants note that to maintain a finding of anticipation, each and every element of the claim must be present in a single prior art reference. Although the Examiner asserts that Tang *et al.* disclose that the claimed polypeptide is expressed in cancer tissue including colon carcinoma, at no point is the specific portion of Tang *et al.* allegedly disclosing such identified. Rather, Applicants submit that at no point do Tang *et al.* specifically disclose the treatment of “colon cancer.”

Further, not only do Tang *et al.* fail to teach the disease of colon cancer, reciting only a genus of gastrointestinal tract cancer, which arguably includes, *inter alia*, mouth cancer, esophageal cancer, stomach cancer, and cancer of the small intestine, but Tang *et al.* also fail to

disclose that the claimed polypeptide is expressed in colon cancer cells. Indeed, Tang *et al.* merely recite hundreds of mechanistically distinct disease conditions, and assert that polynucleotide sequences encoding “ENZM” may be used to diagnose any of the plethora of recited disorders. However, “ENZM” does not even refer to a specific protein, but rather a large genus of functionally and structurally diverse proteins. Accordingly, for the foregoing reasons, Tang *et al.* fail to teach each and every element of Claim 16, as is required to maintain a finding of obviousness.

Further still, Applicants respectfully submit that Tang *et al.* cannot serve as anticipatory prior art at least because it lacks an enabling disclosure. “[T]he disclosure in an assertedly anticipating reference must provide an enabling disclosure of the desired subject matter; mere naming or description of the subject matter is insufficient, if it cannot be produced without undue experimentation.” (Emphasis added.) *Elan Pharm., Inc. v. Mayo Found. For Med. Educ & Research*, 346 F.3d 1051, 1054, 68 USPQ2d 1373, 1376 (Fed. Cir. 2003). In this regard, the disclosure of Tang *et al.* is so generic and non-specific that one of skill in the art would be unable to practice such a method without undue experimentation. By way of example, one of skill in the art would have no indication whatsoever as to what “ENZM,” which may be any one of twelve structurally and functionally unrelated proteins, would be upregulated, or downregulated, if at all, in which of the hundreds of distinct diseases recited by Tang *et al.* Clearly, undue experimentation would be required to practice such a method, absent a specific teaching that the claimed polypeptide is expressed in colon cancers at a different level as compared to normal cells. Accordingly, Tang *et al.* do not anticipate the claims.

Withdrawal of the rejection is respectfully requested.

2. On page 13 of the Office Action, the Examiner rejects Claims 16-18 under 35 U.S.C. 102(a) as being anticipated by Sugiura *et al.* (*Biochem Biophys Res Commun.*, 2004, 27;315(1):204-11, available online January 24, 2004).

In making the rejection, the Examiner alleges that Sugiura *et al.* disclose a gene 100% identical to instant SEQ ID NO: 1, and that the gene is upregulated in colon adenocarcinoma tissue by about 3 fold as compared to normal colon tissue. The Examiner further asserts that Sugiura *et al.* disclose that identification of expression of this gene could be a prognostic marker for colon cancer. Furthermore, the Examiner contends that Sugiura *et al.* disclose a method of detection, such as RT-PCR.

Applicants respectfully submit that the Declaration under 37 CFR 1.132 submitted herewith, showing derivation of the subject matter disclosed in the prior art reference from Applicants, removes Sugiura *et al.* As a legally effective reference.

Withdrawal of the rejection is respectfully requested.

Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

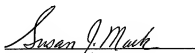
Respectfully submitted,

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

WASHINGTON DC SUGHRUE/265550

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CUSTOMER NUMBER


Susan J. Mack
Registration No. 30,951

Date: August 25, 2008

Wong, L.-J., & Frey, P. A. (1974a) *Biochemistry* 13, 3889-3894.
 Wong, L.-J., & Frey, P. A. (1974b) *J. Biol. Chem.* 249, 2322-2323.

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Site-Directed Mutagenesis of Yeast C₁-Tetrahydrofolate Synthase: Analysis of an Overlapping Active Site in a Multifunctional Enzyme[†]

Charles K. Barlowe,[‡] Mary E. Williams,^{§,¶} Jesse C. Rabinowitz,[‡] and Dean R. Appling^{*-‡}

Clayton Foundation Biochemical Institute, Department of Chemistry, University of Texas, Austin, Texas 78712, and

Department of Biochemistry, University of California, Berkeley, California 94720

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ABSTRACT: C₁-tetrahydrofolate (THF) synthase is a trifunctional protein possessing the activities 10-formyl-THF synthetase, 5,10-methenyl-THF cyclohydrolase, and 5,10-methylene-THF dehydrogenase. The current model divides this protein into two functionally independent domains with dehydrogenase/cyclohydrolase activities sharing an overlapping active site on the N-terminal domain and synthetase activity associated with the C-terminal domain. Previous chemical modification studies on C₁-THF synthase from the yeast *Saccharomyces cerevisiae* indicated at least two cysteinyl residues involved in the dehydrogenase/cyclohydrolase reactions [Appling, D. R., & Rabinowitz, J. C. (1985) *Biochemistry* 24, 3540-3547]. In the present work, site-directed mutagenesis of the *S. cerevisiae* ADE3 gene, which encodes C₁-THF synthase, was used to individually change each cysteine contained within the dehydrogenase/cyclohydrolase domain (Cys-11, Cys-144, and Cys-257) to serine. The resulting proteins were overexpressed in yeast and purified for kinetic analysis. Site-specific mutations in the dehydrogenase/cyclohydrolase domain did not affect synthetase activity, consistent with the proposed domain structure. The C144S and C257S mutations result in 7- and 2-fold increases, respectively, in the dehydrogenase K_m for NADP⁺. C144S lowers the dehydrogenase maximal velocity roughly 50% while C257S has a maximal velocity similar to that of the wild type. Cyclohydrolase catalytic activity is reduced 20-fold by the C144S mutation but increased 2-fold by the C257S mutation. Conversion of Cys-11 to serine has a negligible effect on dehydrogenase/cyclohydrolase activity. A double mutant, C144S/C257S, results in catalytic properties roughly multiplicative of the individual mutations. In addition, the 5' end of ADE3 mRNA was mapped in yeast grown under conditions which repress or derepress C₁-THF synthase transcription. Four major transcription initiation sites are observed in both cases (-27, -30, -38, -42); however, the relative frequency of initiation at these sites differs between the repressed and derepressed states.

In eukaryotes, 10-formyltetrahydrofolate synthetase (EC 6.3.4.3), 5,10-methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), and 5,10-methenyltetrahydrofolate dehydrogenase (EC 1.5.1.5) are present on one polypeptide in the form of a trifunctional enzyme (Paukert et al., 1976, 1977; Tan et al., 1977; Caporilli et al., 1978; Schirch, 1978; de Mata & Rabinowitz, 1980). This enzyme, termed C₁-tetrahydrofolate synthase, is responsible for the interconversion of the one-carbon unit attached to the coenzyme THF.¹ The enzyme from the yeast *Saccharomyces cerevisiae* is similar to the other known eukaryotic C₁-THF synthases, existing as a homodimer of *M_r* = 102 000 (Paukert et al., 1977; Staben & Rabinowitz, 1986).

Several lines of evidence suggest that the eukaryotic enzyme is composed of two functional domains. Proteolysis of the yeast enzyme (Paukert et al., 1977), the porcine enzyme (Tan &

MacKenzie, 1977), or the rabbit enzyme (Villar et al., 1985) results in separation of synthetase activity from the other two activities. In each case, synthetase activity is associated with a large proteolytic fragment (subunit *M_r* = 60 000-80 000), and dehydrogenase and cyclohydrolase activities are associated with a small fragment (subunit *M_r* = 30 000). Schirch (1978) demonstrated coordinate protection by NADP⁺ of the rabbit liver dehydrogenase/cyclohydrolase activities against heat inactivation. In addition, 5,10-methenyl-THF, a product of the dehydrogenase reaction, does not accumulate in the coupled dehydrogenase/cyclohydrolase reaction (Schirch, 1978; Cohen & Mackenzie, 1978; Wasserman et al., 1983). Chemical modification studies with the trifunctional enzyme (Schirch et al., 1979; Smith & Mackenzie, 1978, 1985; Appling & Rabinowitz, 1985a) led to the suggestion of a common active site for the dehydrogenase/cyclohydrolase activities. Experiments with the yeast enzyme (Appling & Rabinowitz, 1985a)

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^{*} To whom correspondence should be addressed.

[‡] University of Texas.

[§] University of California.

[¶] Present address: Rockefeller University, 1230 York Ave., New York, NY 10021.

¹ Abbreviations: THF, tetrahydrofolate; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; bp, base pairs; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride.

are consistent with an overlapping active site and indicate the existence of at least two critical histidyl residues and at least two critical cysteinyl residues at the dehydrogenase/cyclohydrolase active site.

The nucleotide sequence of the ADE3 gene of *S. cerevisiae*, which codes for C₁-THF synthase, has been determined (Staben & Rabinowitz, 1986). The two functional domains of the enzyme have been tentatively mapped onto the nucleotide sequence by correlation with the genetic map for the ADE3 locus (McKenzie & Jones, 1977) and with the proteolysis data. In both the yeast enzyme (McKenzie & Jones, 1977) and the pig liver enzyme (Tan & MacKenzie, 1979), the dehydrogenase/cyclohydrolase domain is N-terminal to the synthetase domain. The junction of the two domains is probably located at or around amino acids 306–321, a stretch that contains six proline residues and several potential protease digestion sites. The putative dehydrogenase/cyclohydrolase domain, encompassing the first 300 or so amino acids, contains 11 histidines, but only 3 cysteines, located at positions 11, 144, and 257 (Staben & Rabinowitz, 1986) (Figure 1). In the present work, we have utilized oligonucleotide-directed mutagenesis to investigate the involvement of these three cysteinyl residues in the overlapping dehydrogenase/cyclohydrolase site. The single amino acid substitutions C11S, C144S, and C257S and the double substitution C144S/C257S were made. The resulting proteins were overexpressed in an *ade3* strain of *S. cerevisiae* and purified by a single-step method. Kinetic analysis of these site-specific mutants is reported in this paper. In addition, the 5' end of the ADE3 transcript has been mapped in yeast grown under conditions which repress or derepress C₁-THF synthase.

EXPERIMENTAL PROCEDURES

Materials. Common reagents were commercial products of the highest grade available. Culture media were obtained from Difco (Detroit, MI). [³²S]-α-Thio-DATP and [γ-³²P]-ATP were purchased from Amersham (Arlington Heights, IL) and ICN (Costa Mesa, CA), respectively. Other radiochemicals were obtained from NEN (Boston, MA). Restriction enzymes were obtained from BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA). Oligonucleotides were synthesized on an Applied Biosystems Model 381A DNA synthesizer, deprotected, and purified by thin-layer chromatography (Alvarado-Urbana et al., 1981). T7 RNA polymerase was prepared as described (Grodberg & Dunn, 1988) from an overproducing strain of *Escherichia coli*, obtained from J. Dunn (Brookhaven National Laboratory). (6*R*,5*S*)-Tetrahydrofolate was prepared by the hydrogenation of folic acid over platinum oxide (Blakley, 1957) and purified by chromatography on DEAE-cellulose (Curthoys & Rabinowitz, 1971). (6*R*)-5,10-Methylen-THF was synthesized enzymatically (Curthoys & Rabinowitz, 1971).

Strains and Growth Conditions. Haploid strains of *Saccharomyces cerevisiae* as used in all studies. M1614C (a *ser1 leu1*) and *ade3-5281* (a *ser1 ura1 ade3-130*) were obtained from E. Jones (Carnegie Mellon University, Pittsburgh, PA). *ade3-5281* has a large deletion at the ADE3 locus. DAY3 (a *ura3-52 ade3-130 trp1 leu2 his3 his4 ser1-171*) was constructed by crossing *ade3-5281* with JRY478 (a *his3 his4 trp1 leu2 ura3-52 str3⁺*) (Sherman et al., 1986). JRY478 was a gift from J. Rine (University of California, Berkeley). Rich media (YPD) contained 1% yeast extract, 2% bacto-peptone, and 2% dextrose. Minimal media (YMD) consisted of 0.7% yeast nitrogen base and 2% dextrose and was supplemented with the following nutrients as needed (final concentration in mg/L): serine (375), leucine (30), histidine (20), tryptophan

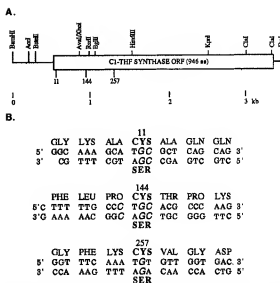


FIGURE 1: (A) A portion of the ADE3 locus showing the C₁-THF synthase open reading frame (ORF) and the 5' and 3' noncoding regions (thin lines). Restriction endonuclease sites referred to in the text are identified. Locations of the three cysteine codons are shown below the map. (B) Amino acid and nucleotide sequences surrounding the three cysteine-to-serine mutations are shown. The upper nucleotide strand (5' → 3') is the sequence of the mp18/HIND template (see Experimental Procedures). The lower nucleotide strand (3' → 5') is the mutagenic oligonucleotide. The mismatches are indicated in large italics.

(20), uracil (20), adenine (20), glycine (20), and formate (1000).

In Vitro Mutagenesis. Figure 1A shows the 2838 base pair open reading frame for C₁-THF synthase and the restriction sites used in constructing site-specific mutants. The mutagenic oligonucleotides for conversion of cysteine codons to serine are seen in Figure 1B. Oligonucleotide-directed mutagenesis was carried out according to Nisbet and Beilharz (1985). The single-stranded template used for all the experiments reported here is referred to as mp18/HIND (refer to Figure 2 for an outline of constructs). It is comprised of the 5'-terminal *Bam*HI-*Hind*III restriction fragment, containing the first 1508 nucleotides of the ADE3 gene, ligated into the multiple cloning site of M13mp18 (Norlander et al., 1983). Following transformation (Hanahan, 1985) of *E. coli* TG-1 (Carter et al., 1985), mutants were detected by hybridization of plaque lifts with mutant oligonucleotide and washing at increasing stringency (Zoller & Smith, 1983). Putative mutants were plaque purified and sequenced. The mutagenized region was removed from the M13mp18 vector by *in vitro* primer extension and restriction digestion as follows (refer to Figure 1). *Bst*EII and *Xho*I were used for the C11S mutation, and the resulting 611-bp fragment was purified and ligated into pYCI-Syn previously cut with the same enzymes to remove the wild-type fragment. [pYCI-Syn was derived from YEpADE3 (Staben & Rabinowitz, 1986) by transfer of the 3194-bp *AccI*-*DraI* fragment into pUC13.1 (Figure 2). pUC13.1 is pUC13 (Messing, 1983) in which the *Hind*III site was removed by digestion with *Hind*III, filling in the recessed ends with the Klenow fragment of *E. coli* DNA polymerase (BRL), and ligation of the resulting blunt ends. This was done to facilitate reconstruction of some of the mutant genes.] For the C144S mutation, the *Xho*I and *Bgl*II sites were used. The resulting 172-bp fragment was used to reconstruct pYCI-Syn as above. For the C257S mutant, the primer-extended vector was digested with *Hind*III and *Bgl*II, and the resulting 469-bp

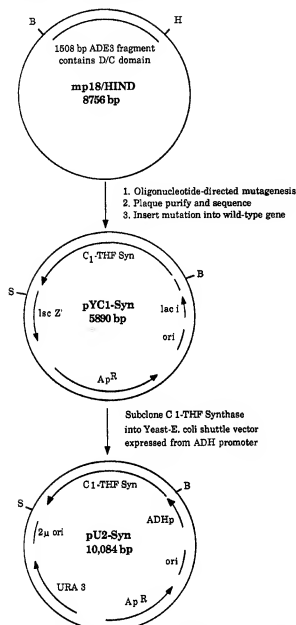


FIGURE 2: Restriction sites are indicated as follows: B = BamHI, H = HindIII, and S = SstI. The single-stranded mp18/HIND contains the BamHI-HindIII fragment of the ADE3 gene encoding the dehydrogenase/cyclohydrolase (D/C) domain. The *AclI*-*DraI* fragment from YEPADE3 (see text and Figure 1A) was blunt ended and ligated into the *SmaI* site contained within the multiple cloning region of pUC13.1 (see text) to produce pYC1Syn. The BamHI and SstI sites of pYC1Syn are derived from the pUC13.1 multiple cloning site. The BamHI-SstI fragment from pYC1Syn, containing the entire C₁-THF synthase ORF, was then transferred into pVT-102U to produce pU2-Syn (see text). Other abbreviations are as follows: ori, *E. coli* origin of replication; Ap^R, ampicillin resistance (β -lactamase gene); 2 μ ori, yeast 2 μ origin of replication; ADHP, yeast alcohol dehydrogenase gene promoter.

fragment was isolated and ligated into pYC1Syn previously out at the same sites. Before the mutagenized fragments were removed from mp18/HIND, the entire fragment was sequenced to ensure that no secondary mutations had occurred. Dideoxy sequencing was performed with [³²S]- α -thio-dATP (Biggin et al., 1983) and either Sequenza (USB, Cleveland, OH) or the Klenow fragment of *E. coli* DNA polymerase. The

mutant pYC1Syn constructs were used to transform *E. coli* SCS1 or XL1-Blue (Stratagene, La Jolla, CA) to ampicillin resistance. The double mutant, C144S/C257S, was constructed by replacing the 802-bp BamHI-RsrII fragment of C257S in pU2-Syn (Figure 2) with the same BamHI-RsrII fragment from C144S.

Expression and Purification of Mutant Enzymes. The yeast shuttle vector pVT-102U (Vernet et al., 1987) was a gift from T. Vernet and was utilized for overexpression of the wild-type and mutant proteins. The 3209-bp BamHI-SstI fragment from pYC1Syn was inserted into the multiple cloning site of pVT-102U to yield pU2-Syn (see Figure 2). The resulting construct was used to transform yeast.

The *ade3* strain of *S. cerevisiae*, DAY3, was transformed to uracil prototrophy with pU2-Syn by a LiCl method (Ito et al., 1983). Transformants were streaked onto YMD plates plus essential nutrients. To ensure that the proper construct had been introduced into DAY3, yeast plasmid DNA was isolated as described (Burke et al., 1983) except that GeneClean (Bio 101, La Jolla, CA) was used to recover plasmid DNA instead of ethanol precipitation. Restriction analysis (the C1IS mutation removes an *SphI* site while the C144S produces a new *Sall* site) and DNA sequencing confirmed the proper constructs of pU2-Syn in DAY3 transformants. The transformed strains express C₁-THF synthase approximately 40-fold over wild-type *S. cerevisiae*.

C₁-THF synthase activity determinations and purification of overexpressed protein were performed essentially as described previously (Staben et al., 1987). Transformed yeast were grown in 3 L of minimal media to an OD₆₀₀ of about 2 and harvested by centrifugation. After being washed with deionized water, cells were resuspended in 2 volumes of buffer A [25 mM Tris-SO₄ (pH 7.5), 10 mM KCl, 10 mM 2-mercaptoethanol, 1 mM PMSF] and passed three times through a French pressure cell at 4 °C. This lysate was centrifuged at 25000g for 1 h, and the resulting supernatant fluid was filtered through cheese cloth and applied to a 2.5 \times 20 cm heparin-agarose column (Davison et al., 1979) equilibrated with buffer A. The column was washed with 400 mL of buffer A followed by 400 mL of buffer A containing 0.1 M KCl. C₁-THF synthase was eluted with a 1 L gradient from 0.1 to 0.8 M KCl in buffer A, and fractions were monitored for 10-formyl-THF synthetase activity. Peak activity fractions were analyzed by 10% SDS-PAGE (Laemmli, 1970), after staining with Coomassie R250. Fractions containing purified protein were pooled and concentrated by dialysis against 50% glycerol (v/v) in buffer A and stored at -20 °C. Typically, 8–12 mg of purified protein was obtained from 10 g wet weight of DAY3 transformed with pU2-Syn.

Enzymatic Assays. 10-Formyl-THF synthetase was assayed under saturating conditions as previously described (Appling & Rabinowitz, 1985b). Initial velocity measurements of 5,10-methylene-THF dehydrogenase were determined by a modified end-point assay (Scrimgeour & Huennekens, 1963). Assays contained in a total volume of 1 mL 50 mM HEPES (pH 7.5) and 100 mM KCl. The dependence of initial velocity on NADP⁺ concentration was made with 0.5 mM (6R,S)-THF, 0.8 mM formaldehyde, 20 mM 2-mercaptoethanol, and varying amounts of NADP⁺. The dependence of initial velocity on 5,10-methylene-THF concentration was determined at 0.6 mM NADP⁺, 10 mM 2-mercaptoethanol, 0.8 mM formaldehyde, and varying amounts of (6R,S)-THF. Reactions were preincubated at 37 °C for 4 min to allow for equilibration of the condensation reaction of THF with formaldehyde to produce 5,10-methylene-THF. The assays were

initiated by the addition of enzyme at 37 °C and terminated after 1 min by the addition of 0.25 mL of a solution containing 1.25 M HCl and 0.075 M 2-mercaptoethanol. Enzymatic activity was calculated from the extinction coefficient at 350 nm of 24 900 M⁻¹ for 5,10-methylene-THF. The equilibrium concentration of 5,10-methylene-THF was calculated by solving the simultaneous equations for the equilibria between THF, formaldehyde, and 2-mercaptoethanol (Kallen & Jencks, 1966). The Michaelis-Menten parameters V_{max} and K_m were determined by a nonlinear regression computer program (Duggleby, 1981). The initial velocity measurements of 5,10-methylene-THF cyclohydrolase activity were obtained by following the decrease in absorbance at 355 nm as described elsewhere (de Mata & Rabinowitz, 1980) except that a final concentration of 50 mM 2-mercaptoethanol was used. Protein concentrations were estimated according to the dye-binding assay of Bradford (1976) with BSA as a standard. Absorbance readings were performed on a Perkin-Elmer Model Lambda 3A spectrophotometer.

Primer Extensions. RNA was isolated from yeast as previously described (Appling & Rabinowitz, 1985b). Poly(A⁺) RNA was prepared according to Aviv and Leder (1972). A synthetic oligonucleotide [5'-CCAACACTTGACCAAGC-3'] was used to hybridize to the first 19 nucleotides of the large open reading frame predicted from the sequence of the ADE3 gene (Staben & Rabinowitz, 1986) was phosphorylated at its 5' end with γ -³²PATP and T4 polynucleotide kinase (BRL) and separated from unincorporated label by chromatography on DEAE-cellulose. Ten micrograms of poly(A⁺) RNA and 0.1 pmol of the labeled oligonucleotide primer were dried in a Speed-Vac and redissolved in 10 μ L of solution containing 50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 0.1 mM EDTA. Annealing was carried out by incubation at 90 °C for 10 min followed by 2 h at 37 °C. Upon transfer to 37 °C, 1 μ L of a solution containing 30 units of RNasin (Promega Biotec) and 100 mM DTT was added. Following annealing, 10 μ L of a solution containing 50 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 10 mM DTT, 1 mM each of dATP, dCTP, dGTP, and dTTP, and 100 units of Moloney-Murine leukemia virus reverse transcriptase (BRL) was added to the primer-template solution. Reaction proceeded at 37 °C and was stopped after 30 min by addition of 1 μ L of 0.5 M EDTA. The sample was extracted with phenol/chloroform and precipitated with ethanol. The pellet was dissolved in 50 μ L of 0.2 M NaCl and reprecipitated with 125 μ L of 95% ethanol. After being rinsed with 70% ethanol, the pellet was dissolved in 10 μ L of loading dye (99% formamide, 0.03% each bromophenol blue and xylene cyanol, and 10 mM EDTA). Samples were heated to 95 °C for 3 min before electrophoresis on an 8% polyacrylamide sequencing gel (Maxam & Gilbert, 1980). Dideoxy sequencing reactions were carried out on the mp18/HIND template with the same oligonucleotide as a primer.

S1 Nuclease Mapping. Mapping of the 5' end of the transcripts was performed as described by Quarles and Heinrich (1986). Single-stranded [³²P]cRNA probe was prepared from pTZ19R/Aval in vitro transcription with T7 RNA polymerase. Transcription reactions were carried out essentially according to protocols provided by Promega Biotec. pTZ19R/Aval was derived from pYC1Syn by subcloning the 847-bp BamHI-Aval fragment into the SmaI site of pTZ19R (Pharmacia). The orientation is such that transcription by T7 RNA polymerase will produce a complementary RNA. pTZ19R/Aval was linearized with BstEII before being transcribed to yield a cRNA of 611 nucleotides.

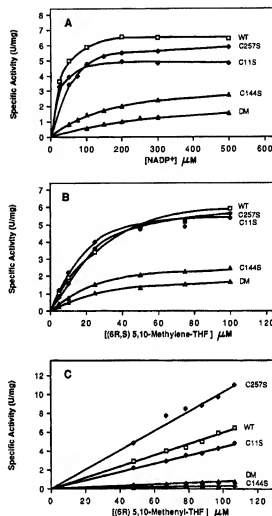


FIGURE 3: Dependence of dehydrogenase enzyme activity on NADP⁺ concentration (A) and (6R,S)-5,10-methylene-THF concentration (B) for wild-type and mutant C₇-THF synthases. (C) Dependence of cyclohydrolase activity on (6R,S)-5,10-methylene-THF concentration. The abbreviation DM represents the double mutant C144S/C257S. Specific activity is expressed as $\mu\text{mol}\cdot\text{min}^{-1}\cdot(\text{mg of purified protein})^{-1}$.

The labeled probe was gel purified before use as described (Quarles & Heinrich, 1986). Samples were analyzed on a 5% sequencing gel with a sequencing ladder for markers.

RESULTS

The pU2-Syn construct allowed for overexpression and purification of wild-type C₇-THF synthase and four site-specific mutants in this trifunctional enzyme. Figure 3 shows the dependence of specific activity on substrate concentration for these enzymes, and kinetic constants calculated from these data are listed in Table I. Substitution of cysteine residues in the dehydrogenase/cyclohydrolase portion of C₇-THF synthase has varying effects on the enzymatic properties of 5,10-methylene-THF cyclohydrolase and 5,10-methylene-THF dehydrogenase. However, none of the substitutions significantly affects the 10-formyl-THF synthetase activity (data not shown), which is catalyzed by a functionally independent domain.

Effect of Mutations on the Catalytic Properties of 5,10-Methylene-THF Dehydrogenase. Mutant C11S has properties very similar to those observed for the wild-type enzyme, with only slight effects on K_m and V_{max} . Replacement of Cys-144

Table 1: Kinetic Constants* for Wild-Type and Mutant C₇-THF Synthase

enzyme	dehydrogenase			5,10-methylene-THF			cyclohydrolase, 5,10-methylene-THF, V ₁₀₀ ^b
	K _m	V _{max}	k _{cat} /K _m	K _m	V _{max}	k _{cat} /K _m	
wild type	23 ± 2	7.2 ± 0.1	1060	31 ± 1	7.6 ± 0.2	834	5.9
C11S	14 ± 1	5.2 ± 0.1	1260	22 ± 2	6.7 ± 0.3	1035	4.4
C144S	172 ± 6	3.7 ± 0.1	73	36 ± 2	3.4 ± 0.1	321	0.3
C257S	46 ± 3	6.6 ± 0.1	488	45 ± 4	8.6 ± 0.5	650	10.3
C144S/C257S	397 ± 42	3.0 ± 0.2	26	42 ± 2	2.4 ± 0.1	194	0.7

* K_m, V_{max}, and k_{cat}/K_m are expressed in μM, μmol·min⁻¹·mg⁻¹, and s⁻¹·mM⁻¹, respectively. k_{cat} is calculated by use of a M_r of 204,000 for C₇-THF synthase (Staben & Rabinowitz, 1986). Kinetic constants and standard deviations were determined by a nonlinear regression computer program previously described by Duggleby (1981). ^b V₁₀₀ = initial velocity in μmol·min⁻¹·mg⁻¹ at 100 μM 5,10-methylene-THF.

with serine has the most marked effect of the single amino acid changes. An increase in K_m for NADP⁺ of approximately 7-fold is observed while V_{max} is reduced roughly 50% for this mutant. The K_m for 5,10-methylene-THF is not significantly different when C144S is compared to the wild-type, and in fact, the dehydrogenase K_m for this substrate is largely unchanged for all mutants that were generated. Substitution of Cys-257 with serine increases the K_m for NADP⁺ by about 2-fold, while having a small effect on the V_{max} of this enzyme. The double mutant, C144S/C257S, has a K_m of about 0.4 mM, a 17-fold increase over that of the wild-type, and also exhibits a 60% decrease in V_{max}.

Effect of Mutations on the Catalytic Properties of 5,10-Methylene-THF Cyclohydrolase. The cyclohydrolase from *S. cerevisiae* has a reported K_m for (6R)-5,10-methylene-THF of 0.5 mM (de Mata & Rabinowitz, 1980). In these experiments, cyclohydrolase activity could not be determined at or near saturating conditions due to the high absorbance of substrate; therefore, accurate values for K_m and V_{max} could not be calculated. However, the specific activity plots clearly reveal dramatic effects on cyclohydrolase activity due to the site-specific substitutions (Figure 3C). C144S shows a decreased specific activity of 20-fold at 0.1 mM (6R)-5,10-methylene-THF compared to the wild-type enzyme. In contrast to C144S, a doubling of specific activity is demonstrated by the C257S mutant. Substitution of both Cys-144 and Cys-257 results in a specific activity plot similar to that of the single Cys-144 replacement but with a slightly higher activity than that of C144S at a substrate concentration of 0.1 mM.

Mapping the 5' End of the ADE3 Transcript. Figure 4 shows an autoradiogram of the products resulting from primer extension of poly(A⁺) RNA isolated from M1614C grown under repressing (YPD) or derepressing (glycine + formate) conditions and from a deletion strain (3-5281). The ADE3 transcripts were 2–3-fold more abundant in RNA from the derepressed strain than from the repressed strain, consistent with results obtained previously from an in vitro translation/immunoprecipitation assay for ADE3 mRNA (Appling & Rabinowitz, 1985b). The same three to four major start sites are seen for M1614C from the two growth conditions. However, the relative intensities of the bands differ between repressing and derepressing conditions. Nearly half (44%) of the ADE3 transcripts detected in RNA from yeast grown in the rich medium (YPD) start at a position 30 nucleotides upstream from the AUG codon. Three other start sites occur with approximately equal frequency at -42 (14%), -38 (19%), and -27 (23%). Transcripts from derepressed yeast start with approximately equal frequency at -42 (29%), -38 (33%), and -30 (27%), with initiation at -27 observed in only 11% of the transcripts. RNA from 3-5281 yields no primer extension products, as expected in this strain carrying a complete deletion of the ADE3 locus. These start sites were confirmed by SI mapping (data not shown).

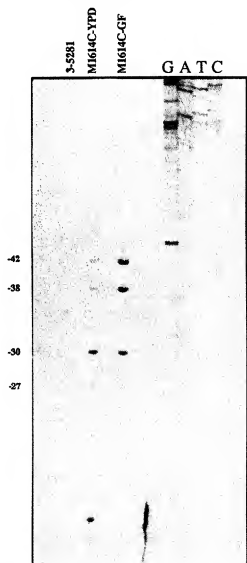


FIGURE 4: Primer extension of ADE3 mRNA. Polyadenylated RNA from yeast strain 3-5281 (ADE3 deletion) or M1614C (wild type) grown in rich medium (YPD) or glycine + formate (GF) was primer extended with a synthetic oligonucleotide complementary to nucleotides +1 to +19 (see Figure 5) as described under Experimental Procedures. A sequencing ladder, generated from mp18/HIND with the same oligonucleotide primer, provided markers. Samples were electrophoresed on an 8% polyacrylamide sequencing gel and autoradiographed. The major start sites are indicated on the left.

DISCUSSION

Several reports on kinetic, structural, and genetic properties of C₇-THF synthase indicate an intimate relationship between



FIGURE 5: Initiation sites of the yeast ADE3 transcript. Sequence at the 5' end of the ADE3 locus showing the major start sites (arrows) identified by extension of an oligonucleotide primer complementary to the sequence *overlined* (+19 → +1). Potential promoter elements, as discussed in the text, are *underlined*.

dehydrogenase and cyclohydrolase activities. Chemical modification of yeast C₁-THF synthase with the sulfhydryl modifying agent NEM revealed at least two critical cysteinyl residues for dehydrogenase/cyclohydrolase activities, whereas synthase activity is quite resistant to NEM (Appling & Rabinowitz, 1985a). On the basis of these results, we made single amino acid substitutions at each cysteine residue in the dehydrogenase/cyclohydrolase domain to probe the function of these residues in enzyme catalysis. Indeed, the results of this study indicate Cys-144 and Cys-257 are important residues as site-specific substitution of either of these amino acids affects both dehydrogenase and cyclohydrolase activities.

A wide range of kinetic parameters has been reported for C₁-THF synthase activities from different sources. Dehydrogenase K_m values of 100 μ M for NADP⁺ and 38 μ M for 5,10-methylene-THF have been reported for the *S. cerevisiae* enzyme (de Mata & Rabinowitz, 1980). We report dehydrogenase values somewhat different from those (23 μ M for NADP⁺ and 31 μ M for 5,10-methylene-THF); however, the two determinations were performed under different conditions. Cys-144 appears to be a critical amino acid for both dehydrogenase and cyclohydrolase activity. The rather conservative substitution of this residue with a serine results in a 7-fold increase in dehydrogenase K_m for NADP⁺ and a 50% decrease in V_{max} . Further, the cyclohydrolase specific activity plot for C144S shows an even more dramatic decrease in this activity. An apparent greater sensitivity of cyclohydrolase to this substitution is consistent with previous data showing that NEM inactivated both dehydrogenase and cyclohydrolase but with cyclohydrolase activity at least an order of magnitude more sensitive to this modifying agent than dehydrogenase (Appling & Rabinowitz, 1985a). The dehydrogenase K_m for 5,10-methylene-THF is not significantly affected by this mutation. Our results imply that Cys-144 is important in binding NADP⁺ at the active site, and this idea is further supported by the observation that NADP⁺ protects dehydrogenase and cyclohydrolase activities against sulfhydryl inactivation in the yeast (Appling & Rabinowitz, 1985a) and the rabbit (Schirch et al., 1979) enzymes. Replacement of Cys-257 with serine also affects the catalytic properties of both dehydrogenase and cyclohydrolase. The K_m for NADP⁺ is doubled while only slight changes are seen in K_m for 5,10-methylene-THF and maximal velocity. Interestingly, this mutation doubles the cyclohydrolase specific activity compared to that of wild type at a 100 μ M concentration of 5,10-methylene-THF. Enhancement in catalytic efficiency of other enzymes as a result of site-specific mutations has been previously reported (Carter et al., 1984; Estell et al., 1986). In studies on the chicken liver enzyme, dehydrogenase/cyclohydrolase activities are efficiently coupled such that substrate from the dehydrogenase is channeled between the cyclohydrolase and dehydrogenase with negligible cyclohydrolase

activity toward exogenous 5,10-methylene-THF (Wasserman et al., 1983). Other investigators have found that NADP⁺ activates the rabbit cyclohydrolase (Schirch, 1978) or acts as a competitive inhibitor of the porcine activity (Tan et al., 1977). It has also been suggested that a conformational change in the protein allows for this channeling to occur (Appling & Rabinowitz, 1985a). Specific activity measurements in this paper utilized unchanneled 5,10-methylene-THF; hence, the dramatic decrease in activity of C144S and increase in activity of C257S may reflect changes in the accessibility that this substrate has to the cyclohydrolase catalytic site. Analyzing the effect that these site-specific mutations have on dehydrogenase/cyclohydrolase coupling should contribute to our understanding of this process.

We also constructed C144S/C257S, containing amino acid replacements at both Cys-144 and Cys-257. This enzyme exhibited the lowest catalytic efficiency with an increase in K_m for NADP⁺ of 17-fold and a maximal velocity only 40% that of the wild type. This K_m for NADP⁺ is roughly multiplicative of the 7- and 2-fold increases in K_m observed for the individual mutations. The slight increase in cyclohydrolase specific activity of C144S/C257S over C144S may again reflect a combination of inactivation due to C144S and 2-fold stimulation in activity due to C257S.

Our results are consistent with genetic evidence for the intimate nature of the dehydrogenase/cyclohydrolase activities. McKenzie and Jones (1977) analyzed several point mutants of the ADE3 gene deficient in one or more of the three activities of C₁-THF synthase. All mutants which were deficient in dehydrogenase activity also lacked cyclohydrolase activity (McKenzie & Jones, 1977; Kapor, 1982). Similarly, we find that site-specific mutations which effect one activity also alter the other. In contrast to an ADE3 deletion mutant, the point mutants described by McKenzie and Jones did not have an absolute requirement for adenine, but growth was stimulated by addition of this nutrient. The site-specific mutants which we generated can also grow on minimal media lacking adenine (data not shown). However, caution should be taken in comparing these transformed strains, which overexpress C₁-THF synthase from multicopy plasmids, with strains containing a single copy of the gene.

Recently, the gene for mitochondrial C₁-THF synthase from *S. cerevisiae* has been sequenced (Shannon & Rabinowitz, 1988). The predicted amino acid sequence of mitochondrial C₁-THF synthase shares a striking 71% identity with the cytoplasmic enzyme. In addition, Hum et al. (1988) have isolated and sequenced the cDNA for human C₁-THF synthase. The deduced amino acid sequence for the human protein shares a 58% amino acid identity with yeast cytoplasmic C₁-THF synthase. The region around and including Cys-144 is highly conserved between the three proteins (Figure 6A). The amino acid sequence around Cys-257 is also highly conserved al-

A

HU: F I P C T P K G C I E L I (144-150)
 ADE3: F I P C T P K G I I E L L (141-153)
 MIS1: F I P C T P K G C m k L L (172-184)

B

HU: V I D e G I N Y V P D d k K p n G r K v G D V (233-256)
 ADE3: V I D V G i N Y V a d S K K S G f K e v G D V (238-261)
 MIS1: V I D V G I N Y V P D I S K S G g K I V G D V (261-284)

FIGURE 6: Amino acid sequences of human (HU), yeast cytoplasmic (ADE3), and yeast mitochondrial (MIS1) C₁-THF synthases surrounding cysteine-144 (A) and cysteine-257 (B). Capital letters indicate residues conserved among two or more proteins, and all cysteines are shown in bold face.

though a cysteine is found at that position only in the yeast cytoplasmic C₁-THF synthase (Figure 6B). It is interesting to note that both the human and yeast mitochondrial proteins have a second cysteine five residues away from the conserved Cys-144 while the yeast cytoplasmic protein does not. Cys-11 is not conserved, nor is there appreciable amino acid identity in this region, which may explain the minimal effect mutation of this residue has on dehydrogenase/cyclohydrolase properties.

It is difficult to provide extensive active site information without a three-dimensional structure. However, our results are consistent with the involvement of Cys-144 and Cys-257 in binding and/or positioning of substrate(s) at the active site. The fact that Cys-144 and Cys-257 are not essential for enzyme activity argues against the direct electronic involvement of these residues in catalysis since the chemical reactivities of the oxygen atom in the hydroxyl group of serine and the sulfur atom in the thiol group of cysteine are very different. Also, the possibility exists that these mutations distort the polypeptide structure although this is unlikely since serine and cysteine have a similar spatial arrangement. The facile overexpression and purification of yeast C₁-THF synthase make attempts to crystallize this protein possible, and we hope to obtain x-ray diffraction data on the wild-type and mutant proteins to answer some of these questions and elucidate the mechanistic detail of the trifunctional activities. The identification of cysteinyl residues 144 and 257 as important amino acid residues for dehydrogenase/cyclohydrolase activity contributes to the understanding of this overlapping active site.

Structure of the ADE3 Transcript. Transcription initiation sites at -42, -38, -30, and -27 (+1 defined as the first base in the initiator codon) identified by primer extension are consistent with the existence of consensus sequences for promoter elements within the 5' noncoding region (see Figure 5). A poly(dA-dT) region between -194 and -172, containing 17 T residues in the coding strand, is similar to sequences which serve as upstream promoter elements for constitutive expression in at least three other yeast genes (Struhl, 1985). If this element is serving the same function in the ADE3 gene, it may control expression from the -30 site, since the frequency of initiation at that site showed little or no change during derepression. The observed initiation sites place the best candidate for the TATA element at -100, as the distance between TATA element and the RNA start site averages 60 bp for yeast genes (Struhl, 1986).

Heterogeneity in the 5' end of yeast transcripts is common (McIntosh & Haynes, 1986; Zalkin et al., 1984; Zalkin & Yanofsky, 1982), and in a few cases difference in the relative frequencies of initiation from multiple sites is observed under various conditions (Struhl, 1986). The physiological significance, if any, of changes in the frequency of utilization of the ADE3 start sites under repressed versus derepressed conditions is unknown. These primer-extension experiments confirm and extend our previous results on the regulation of expression of the ADE3 gene. Previously, an *in vitro* translation/immu-

noprecipitation assay to measure C₁-THF synthase mRNA levels demonstrated a 2-3-fold increase upon switching the yeast to derepressing growth conditions (Appling & Rabinowitz, 1985b). A 2-3-fold increase in ADE3 transcript is also observed with the primer-extension assay. It appears that constitutive expression (YPD medium) occurs primarily from the -30 site, since the absolute amount of initiation at this site is unchanged upon derepression. The increase in amount of ADE3 transcript upon derepression is primarily due to increased initiation at the -42 and -38 sites.

ACKNOWLEDGMENTS

We thank Mallory Barcus for her expert technical assistance and Robert MacKenzie for sharing the human C₁-THF synthase sequence prior to its publication.

Registry No. NADP, 53-59-8; [γ -³²P]ATP, 2964-07-0; Cys, 52-90-4; THF synthase, 73699-18-0; 5,10-methylene-THF dehydrogenase, 9029-14-5; 5,10-methylene-THF cyclohydrolase, 9027-97-8; synthetic oligonucleotide, 118436-82-1; 5,10-methylenetetrahydrofolic acid, 3432-99-3.

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α -Secondary Isotope Effects in the Lipoygenase Reaction

Jeffrey S. Wiseman*

Merrell Dow Research Institute, 2110 East Galbraith Road, Cincinnati, Ohio 45215

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ABSTRACT: Isotope effects for the oxidation of [5,6,8,9,11,12,14,15- ^3H]arachidonic acid catalyzed by soybean lipoygenase and by 5-lipoygenase were measured. This labeling pattern represents substitution at each of the vinylic hydrogens of the substrate. The observed isotope effect for soybean lipoygenase was 1.16 ± 0.02 and for 5-lipoygenase was 1.11 ± 0.05 . These isotope effects are inconsistent with any change in hybridization (sp^2 to sp^3) at the vinylic carbons prior to or during the rate-determining step and are concluded to be most consistent with the formation of a carbanion-like intermediate or transition state. In contrast, the oxidation of arachidonic acid by Ce(IV) , which is thought to proceed via a cation radical intermediate, exhibited at most a small isotope effect (1.02 ± 0.01). The reduction potential for the cation radical formed from arachidonic acid in this reaction is estimated to be 2.7 V vs NHE by comparison of the rates of oxidation of arachidonic acid and cyclohexene by Ce(IV) . This is similar to the potential for the cation radical of 2-butenone. No isotope effect (1.00 ± 0.03) was observed in the 5-lipoygenase reaction for conversion of the initially formed product 5-hydroperoxyeicosatetraenoic acid to the epoxide leukotriene A_4 . From this it is concluded that there is little carbon-oxygen bond formation prior to or during the rate-determining step for epoxide formation.

The kinetics and mechanism of soybean lipoygenase have been extensively studied with the expectation that the results will be generalizable to other enzymes of this class, and the available information has been collected in several recent reviews (Schewe et al., 1986; Kühn et al., 1986; Lands, 1984;

Veldink et al., 1984). The enzyme is a monomer with a single iron per active site, which is neither a heme nor an iron-sulfur center. The iron is considered to be the oxidant that oxidizes the 1,4-diene of a polyunsaturated fatty acid to a pentadienyl radical intermediate. The pentadienyl radical is then trapped by oxygen to give a lipid hydroperoxide as the final product. For example, when the substrate is arachidonic acid, as in this study, the product is 15-HPETE.¹ A pentadienyl radical

* Address correspondence to the author at Glaxo Research Laboratories, 5 Moore Drive, Research Triangle Park, NC 27709.